



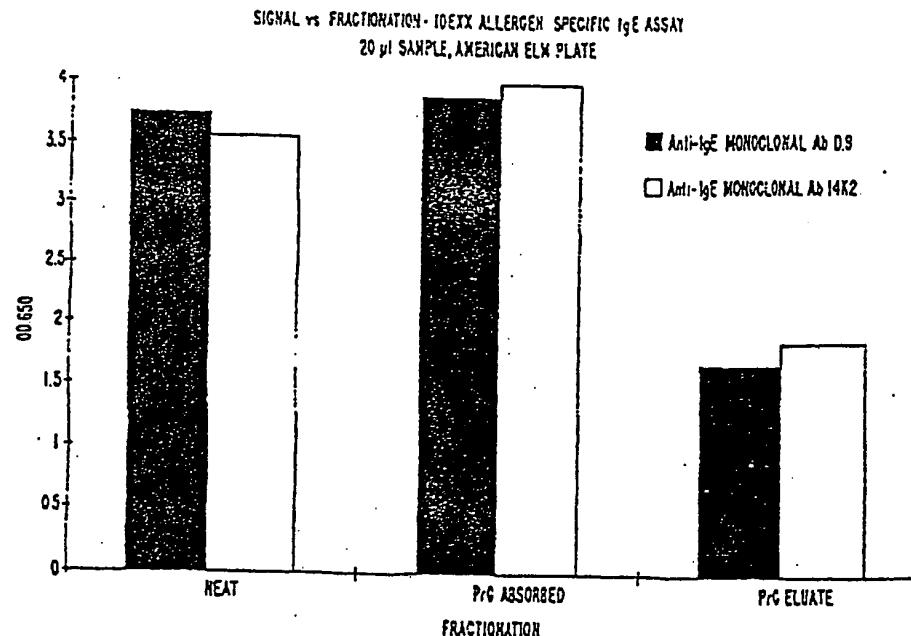
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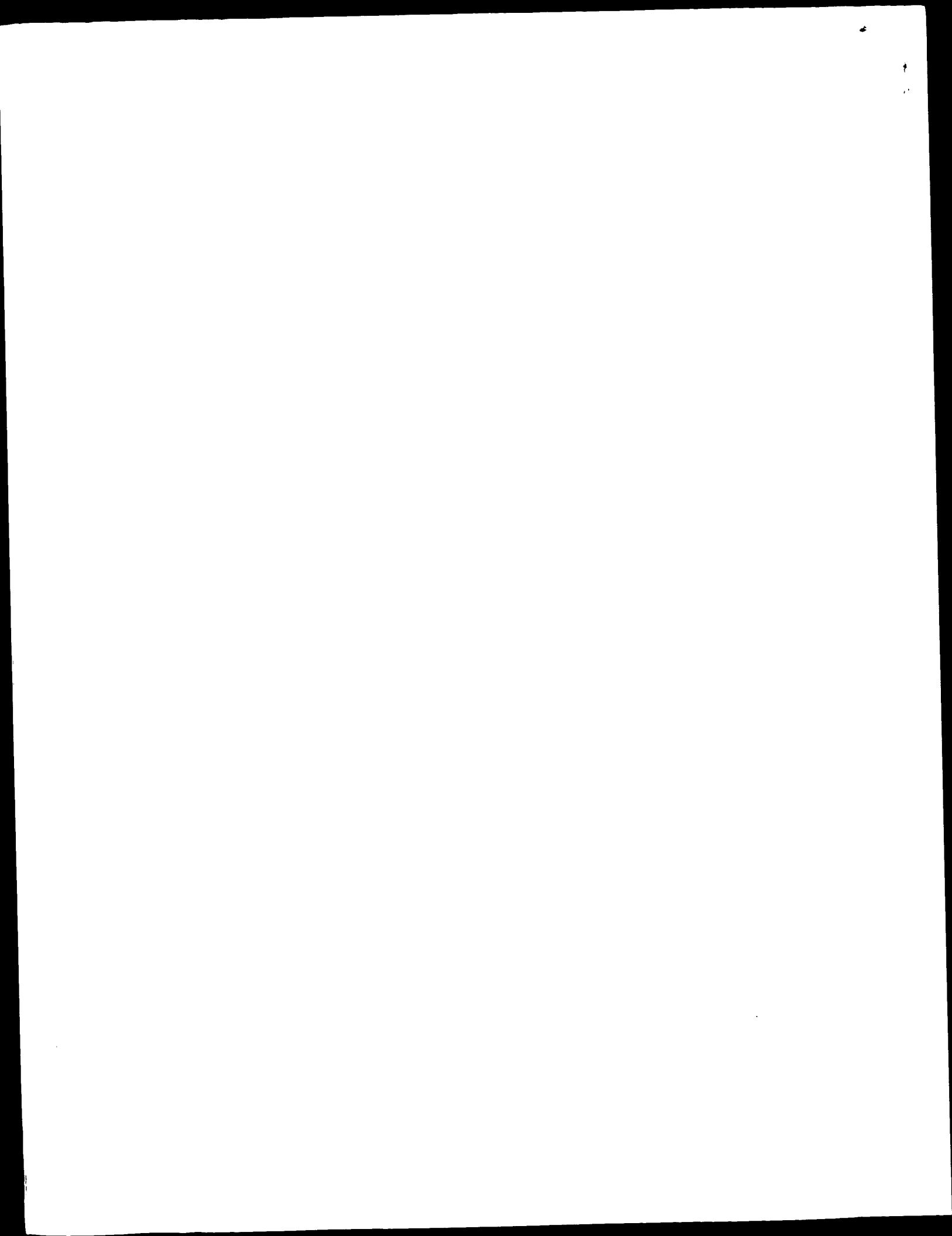
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(54) Title: METHODS AND COMPOSITIONS CONCERNING ALLERGY IN DOGS



(57) Abstract

Compositions such as specific binding proteins and their use with regard to allergy in dogs. Use of the compositions of the invention can facilitate the diagnosis of allergic disease in dogs, particularly where the composition comprises specific binding proteins sensitive and specific to canine IgE, whereby allergen-specific IgE are identified.



METHODS AND COMPOSITIONS CONCERNING ALLERGY IN DOGSFIELD OF THE INVENTION

5 Broadly, this invention relates to methods and compositions which concern pathology in mammals. More specifically, it concerns methods and compositions relevant to allergy in dogs, comprising use of specific binding proteins such as monoclonal antibodies specific to canine 10 IgE.

BACKGROUND ART

15 Approximately one-in-five dogs in the United States has allergies. The most common sign of allergies in dogs is severe scratching; this scratching may result in rashes, hair loss, and secondary infections. Respiratory manifestations, a common mode of allergic reaction in humans, is for unknown reasons a much less common allergic manifestation in dogs.

20 Allergy to fleas is believed to be the most common dog allergy. Typically, a flea's saliva is the allergen, and a single flea bite can cause substantial itching. An additional form of allergy in dogs is termed atopy. Atopy is a condition where a dog is allergic to inhalants such as pollens, molds or microscopic mites found in house dust.

25 Heretofore, when a dog exhibited pruritic dermatitis, the diagnosis has been made in accordance with the following general steps. First, the veterinarian would obtain a history, then do a physical examination of the dog. Frequently, dogs experiencing an allergic reaction have 30 bilaterally symmetrical pruritic disease. Second, a flea examination was done both visually and by flea combing. Flea

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allergy typically produces a predominantly caudal and dorsal distribution. Next, skin scrapings were done to test for Sarcoptes or Cheyletiella mites, and deep skin scrapings for Demodex mites. Additionally, fungal cultures were carried out to rule out dermatophytosis such as resulting from Microsporum or Trichophyton infection. The dog was also evaluated for bacterial skin infection. Typical bacterial skin infection lesions of staphylococcal pyoderma are red papules, pustules and epidermal collarettes. Cytology of intact pustules or moist areas may be diagnostic. Bacterial skin infections were treated with an appropriate antibiotic, continued for two weeks following clinical resolution. Additionally, the dog was evaluated for yeast dermatitis. Smears or scrapping of moist or greasy areas would be examined for Malassezia. Even if lab studies did not indicate yeast infection, treatment for yeast dermatitis would be implemented if such infection was indicated by clinical diagnosis.

Although many of the foregoing diagnostic steps may identify a cause of dermatitis, the possibility exists that such infections (for example, fungal, bacterial or yeast) are secondary infections facilitated by skin trauma brought on by scratching due to allergic reactions. Thus, a positive examination for any of these skin infections does not definitively rule out an accompanying allergic etiology.

Heretofore, to diagnose and treat allergy in dogs the following steps were typically required: If the dog had been placed on steroids to treat symptoms, the animal was withdrawn from steroids for six (6) weeks. Dilutions of various allergen extracts were prepared and loaded into syringes; typically 50 or more allergens were prepared. The

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dog would be sedated, then an area of the dog's flank was shaved which was sufficiently large to receive the 50 or more injections of allergens. Each injection site was marked on the animal with a felt marker and 0.1% ml volumes of an extract were injected intradermally at each site. Reactions were allowed to proceed for 15 to 20 minutes. The sizes of the reactions at the injection sites were compared to positive (histamine) and negative (the allergen diluent) control sites. Unfortunately, the difficulties with this procedure relate to the ability to find an area of skin large enough on an animal to test; this difficulty is especially problematic since the canine manifestation of allergic disease is typically cutaneous. The pigmentation of dog's skin made the reading of the results difficult. Moreover, the differences in skin texture, thickness and toughness from one animal to another made uniform reading of results difficult. In addition, these diagnostic steps suffered from the fact that they were frequently very time consuming.

In mammals, antibody molecules are classified into various isotypes referred to as IgA, IgD, IgE, IgG, and IgM. Antibody molecules consist of heavy and light chain components. The heavy chains of molecules of a given isotype have extensive regions of amino acid sequence homology, and conversely have regions of difference from antibodies belonging to other isotypes. The shared regions of the heavy chains provide members of each isotype with common abilities to bind to certain cell surface receptors or to other macromolecules such as complement, and therefore to activate particular immune effector functions. Accordingly, separation of antibody molecules into isotypes also serves to separate the antibodies according to a set of effector

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functions that they commonly activate. In humans and dogs, IgE is involved in allergy, and recognizes antigen in immediate hypersensitivity reactions.

Monoclonal antibodies having differing degrees of sensitivity to canine IgE and IgG have been identified. (DeBoer, et al. Immunology and Immunopathology 37:183-199 (1993).) DeBoer, et al. identified several monoclonal antibodies which had cross reactivity between IgG and IgE. (See, e.g., DeBoer, et al., Table 3 and accompanying text.) Three monoclonal antibodies (A5, D9, and B3) were identified by DeBoer et al., as having some affinity for canine IgE. Of the monoclonal antibodies identified in DeBoer et al., the antibody D9 appeared to have the greatest degree of neutralization of Prausnitz-Kustner reactivity for atopic dog serum. In the context of canine allergy, DeBoer et al. proposed use of their monoclonal antibodies: in the use of antigen-specific IgE ELISA, and for quantitation of canine IgE; additionally they proposed use for immunostaining of Western Blot assays, to evaluate the molecular specificity of IgE antibodies, as well as for *in vitro* studies on degranulation of mast cells.

In humans the serum level of total IgE is diagnostic of allergic disease. To explore the possibility that the serum level of IgE might also be diagnostic of allergy in dogs, DeBoer performed additional studies. (Hill and DeBoer Am. J. Vet. Res. (July 1994) 55(7):944-48) Publications following the 1993 DeBoer article used monoclonal antibody D9 in an ELISA assay with the following configuration: D9 was bound to a substrate, antibodies were captured by D9 and then D9 having a marker was used to flag the captured antibody.

Subsequent to the invention of the present invention, the Hill and DeBoer ELISA was used to establish the total amount of IgE in canine serum in an effort to diagnose canine allergy. However, it was found that quantifying IgE was of 5 no use whatsoever in the diagnosis of allergy in dogs. (See, e.g., Abstract and Discussion Sections of Hill and DeBoer) This finding was in direct contrast to the situation in human immunology. This result points out the difficulty of any attempt to correlate data between animals of two different 10 genera. This difficulty is further exemplified by the fact that dogs can be allergic to a different set of antigens than humans are. Fleas, for instance, are a severe problem for dogs but not humans. Furthermore, in instances where dogs and humans appear to be allergic to the same allergen 15 extract, studies by doctors Esch and Grier of Greer Laboratories, have indicated that the specific allergens in an allergen extract which produce canine disease are not necessarily the same allergens that produce disease in humans. For example, it is known that the immunodominant 20 components of dust mite extracts are different in dogs than in humans.

Moreover, the use of monoclonal antibodies directed to canine IgE in a context of diagnosis or treatment of allergy in dogs has been problematic for a number of reasons. 25 Monoclonal and polyclonal antibodies will have different affinities for a given target, and the affinity of a monoclonal composition will be lower. A general quality of monoclonal antibodies, as distinguished from polyclonal antibodies, is that a monoclonal antibody due to the limited 30 antigenic determinant to which it binds has a lower overall affinity to a given target; this lower affinity correlates to

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a lower sensitivity for monoclonal antibodies as compared to polyclonal antibodies. The polyclonal serum will possess the possibility of binding to a number of epitopes on a target and thereby generate a test result of higher sensitivity.

5 Accordingly, in the veterinary art it is understood that the use of monoclonal antibodies in assays such as concerning the immunology of allergic disease is discouraged, due to the inability of monoclonal antibodies to produce effective assay results (Oral Communication from

10 Biomedical Laboratories, Inc.; Greer Laboratories, Inc.; and Schwartzman, Inc.). This understanding in the art concerning monoclonals will be altered consequent to the results of the present invention.

15 DISCLOSURE OF THE INVENTION

Disclosed is a specific binding protein that specifically and/or sensitively binds to canine IgE. In preferred embodiments the specific binding protein comprises a monoclonal antibody D9, 14K2, 1B1, or 11B11. The specific binding protein can further comprise a means for producing a signal. In accordance with the invention, a plurality of specific binding proteins can comprise a polyclonal serum reactive to canine IgE.

25 Disclosed herein is a method to assay for the presence of allergen-specific canine IgE, said method comprising steps of obtaining serum or plasma from a dog; contacting the serum or plasma with a canine allergen so that allergen-specific canine IgE become bound to the allergen; then introducing to the allergen, specific binding proteins that sensitively and specifically bind to canine IgE, said specific binding proteins comprising a means for producing a signal; and,

identifying the presence of the signal, whereby the presence of the signal indicates the presence of canine IgE bound to the allergen.

Disclosed is a method to assay for the presence of allergen-specific canine IgE, said method comprising steps of obtaining serum or plasma from a dog; contacting said serum or plasma with a canine allergen immobilized on a solid phase whereby an IgE in the serum will bind to the allergen; washing nonbound material from the allergen; contacting the allergen with a specific binding protein that comprises a means for signaling, wherein the specific binding protein specifically binds canine IgE; and, evaluating for the presence or absence of the means of signaling of the specific binding protein bound to the canine IgE bound to the allergen, so as to identify the presence of allergen-specific canine IgE.

Disclosed is a method to assay for the presence of allergen-specific canine IgE, said method comprising: obtaining serum or plasma from a dog; providing a solid phase flow matrix capable of driving fluid movement, said matrix comprising a region which comprises immobilized canine allergen; providing a mobile disclosure reagent which can move through the matrix, wherein the mobile disclosure reagent comprises specific binding proteins that specifically bind canine IgE; providing a washing liquid which can move through the allergen immobilized on the solid phase matrix; contacting the allergen with the mobile disclosure reagent, wherein said disclosure reagent is detectible; removing material nonbound to the allergen from the region comprising immobilized allergen by contacting the allergen with the washing liquid; and, detecting the presence of the disclosure

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reagent attached to the canine IgE which is attached to allergen, thereby identifying allergen specific canine IgE. The method can comprise that the step of providing a matrix provides a solid phase flow matrix comprising two matrix regions: (i) a region comprising immobilized canine allergen; and, (ii) a receipt region, said receipt region comprising a region for receipt of the serum or plasma, and a region for receipt of the mobile disclosure reagent and receipt of the washing liquid. The method can provide that the step of providing the washing liquid is the step of providing the mobile disclosure reagent.

Disclosed is a method to facilitate diagnosis of allergy in a dog, said method comprising: physically examining the dog; extracting serum or plasma from the dog; performing an assay on the serum or plasma to sensitively and specifically identify the presence of allergen-specific canine IgE within 300 minutes of initiation of performance of the assay; and, identifying the presence of allergen-specific canine IgE.

19. The method of claim 18 wherein the identifying step comprises materials in the following orientation: a solid phase, an allergen adherent to the solid phase, canine IgE specifically bound to the allergen, and, a specific binding protein comprising a means for signaling specifically bound to the canine IgE.

25 Disclosed is a kit for use to facilitate the diagnosis of allergy in dogs, said kit comprising: a solid phase material comprising a canine allergen in contact therewith; and, specific binding proteins conjugated with a means for signaling.

DESCRIPTION OF FIGURES

Fig. 1 illustrates data from use of assays in accordance with the invention using monoclonal antibodies D9 (solid bars) or 14K2 (open bars), where 20 μ l of the serum of Patient 5 One was used in an assay without modification (neat; following adsorption to Protein G (PrG adsorbed); and the results from use of the eluates of the protein G column.

MODES FOR CARRYING OUT THE INVENTION

10

Preparation of Flea Allergens

Flea allergens were made from batches of 5 g of whole fleas which were frozen. The fleas were ground with a chilled mortar and pestle. An extraction buffer was prepared

15 consisting of 10X Dulbecco's PBS (5.0 ml); Leupeptin 1.0 mg/ml (0.5 ml); aprotinin 20 TIU/ml (0.5 ml); 100 mM PMSF (0.5 ml); Iodoacetamide (0.09 g); and dH₂O (QS to 50 ml). A small amount of the extraction buffer was added to the ground fleas and was worked to form a paste. This was repeated

20 until 75% of the extraction buffer was added. The material was then transferred to a beaker containing a stir bar. The remaining extraction buffer was used to rinse the mortar and pestle into the beaker. The beaker was then stirred for 48-72 hours. The flea suspension was then passed through

25 loosely packed glass wool with the extract collected in a clean container. The filtrates were then centrifuged at 35,000 rpm in a Beckman Ti70.1 or Beckman Type 35 rotor (Beckman, Palo Alto), for 30 minutes. The filtrate was then filtered under aseptic conditions through 0.45 μ m low protein-binding filter, followed by filtering through a 0.2 μ m low protein-binding filter. The extract can then be

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stored at -20°C. Low molecular weight material was removed by gel filtration. The crude extract was filtered through a 0.2 μ m filter and then run over a G-25 column. Material was collected from beginning to end of the first peak to elute
5 from the column (exclusion volume). The purified flea extract was concentrated by ultrafiltration through a 3,000 MWCO membrane (Amicon, Beverly, MA) of a size chosen to correspond to a volume of 1/10 to 1/20 of the initial volume. The purified extract was concentrated approximately 10-20 times.
10 The material was then filtered through a 0.2 μ filter into a sterile container.

Preparation of Pollen, Mold and Dust Mite Allergens

The steps for preparation of pollen, mold and dust mite allergens were all conceptually similar. Allergen extracts were obtained from commercial sources (e.g., Greer). Assays in accordance with the invention comprised use of allergens from: Cottonwood, Elm, Box elder, Red Maple, Red Oak, Virginia Live Oak, Bermuda Grass, KBG, Orchard Grass, Timothy, Yellow Dock, Lamb's Quarters, Pigweed, Giant Ragweed, Ragweed, Alternaria, Aspergillus, Helminthosporium, Hormodendrum, Penicillium, and D. Farinae. For each 50 ml of allergen to be processed, a 1 m length of Spectrapor 3 (Spectrum Medical Industries, Houston) (3,500 MWCO, 18 mm
20 diameter) tubing was cut. The tubing was soaked in four washes of 2 liters of deionized water over one hour. The inside of each piece of tubing was then rinsed with deionized water and the bottom was tied off; allergen was loaded into the tubing leaving room for sample volume expansion. The top
25 of each tubing was tied off. The samples were dialyzed with magnetic stirring with four changes (6 to 18 hours each) of
30

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PBS. The allergen sample was collected by transferring it from the tubing (Nalge Co., Rochester) to a 150 ml, 0.45 μ m (CN) filter unit, filtering and then storing at 4°C.

5 Thereafter, the allergen extract was filtered through 0.22 μ m (Millex-GV/syringe filter, Millipore, Bedford) into an ultra filtration unit, and then-concentrated at 50 psi (nitrogen) on a 3000 MWCO membrane to a final volume of 5-10 ml. The allergen concentrate was then filtered at 0.2 μ m (Millex-GV/syringe filter) into a sterile polypropylene tube 10 and stored until further use.

15 Mean protein concentration of the allergen concentrate was determined by BCA assay (Pierce, Rockford). Preferably, mean protein concentration should be greater than 10 mg/ml; if less, further concentration was carried out.

Coating of Solid Phase Material with Concentrated Allergens

20 In a preferred form of the invention allergens are in contact with a solid phase. The solid phase can be a fluid impermeable solid surface such as polystyrene, metal or glass, or a porous matrix. In a preferred embodiment, the allergens are in contact with solid phase particles such as 0.8 micron microspheres. The methods for coating of microspheres with pollen, flea, dust mite and mold allergens were all analogous.

25 The allergens were first non-specifically adsorbed to the surface of microparticles. The particles were 0.8 micron carboxylated polystyrene. (Seradyn, Indianapolis). Microparticles were available with different concentrations 30 of carboxyl groups on their surfaces, referred to as acid content. High acid content particles were used. The allergens were then covalently linked to the particles by use

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of EDAC, to form an amide linkage. Accordingly, the amino groups of the allergens were linked to the carboxyl groups on the microparticles.

A buffer of N-morpholino ethane sulfonic acid (MES) 0.5 M, pH 5.5, 50 ml was prepared according to standard methodologies. N-morpholino propane sulfonic acid (MOPS) 0.5 m, pH 7.2 was prepared according to standard methodologies. 2% BSA in 0.05 m MOPS pH 7.2/0.5% kathon were prepared by standard methodologies; 7.5% sucrose in 0.05 m MOPS pH 7.2/0.5% kathon was prepared according to standard methodologies and was filtered at 0.2 μ m into a sterile container. All buffers were 0.2 μ m filter.

Allergen concentrates were prepared as addressed herein. Polystyrene microparticles were obtained from commercial sources such as (Seradyn, Indianapolis). The final coating concentration for pollen allergens was between 1 mg/ml to 5 mg/ml, mold allergen concentrate reagents were typically performed at a final coating concentration of 5 mg/ml, as were dust mite allergens; all at 3% particle solids. The calculated amount of allergen needed to coat at the desired concentration and the amount of polystyrene particles to add to achieve 3% final solids were determined in accordance with standard methodologies. The MES pH 0.5 m was added at an amount equal to one-tenth of the mls of the coating reaction. The amount of 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride (EDAC) (Pierce, Rockford) was determined as follows:

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2.5 x (μ mol/mg acid content particles)52 UMOL/ml EDAC stock

x

5 (30 mg particles x ml(s) coating reaction) = ml 10 mg/ml EDAC stock to add.

10 The mls of deionized water to add was calculated as follows: the amount of mls of the coating reaction, less the mls of the allergen, less the mls of the particles, less the mls of MES, less the mls of EDAC equals the needed mls deionized water.

15 To coat the 0.8 micron polystyrene particles, the calculated amount of 0.5 M MES pH 5.5 stock was added to a tube. The calculated amounts of deionized water and particles were added to each tube and briefly mixed. The calculated amount of allergen concentrate was added to each tube and the tube then sealed tightly. The tube was incubated with end-over-end mixing for 15 minutes at room temperature. Thereafter, the 10 mg/ml EDAC stock was prepared and immediately added to each tube. Incubation with end-over-end mixing was done for 2 hours at room temperature.

20 25 Each coated particle prep was transferred to a polypropylene centrifuge tube (Sarstadt, Newton, NC); each original tube was rinsed with 3 reaction volumes of 0.05 M MOPS pH 7.2 and the rinse was added to its respective new tube.

25 30 Centrifugation was carried out at 10,000 rpm for 15 minutes in a Beckman JA 20 rotor; the resulting supernatants were decanted and 8 ml 0.05 M MOPS pH 7.2 was added to each tube. The particle pellets were sonicated with a probe sonicator (Branson, Danbury, CT) in accordance with standard

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methodologies in order to re-disperse the particles. The centrifugation, decanting, addition of MOPS and sonication were performed thrice. The resulting products were centrifuged at 10,000 rpm for 15 minutes, the washed particles collected and the supernatant decanted. One reaction volume (the batch size) of 2% BSA and 0.05 m MOPS pH 7.2/0.5% kathon was added to block each particle preparation. Sonication was performed to resuspend the pellets. The tubes were incubated in a rack with orbital mixing for 1 hour at room temperature or overnight at 4°C. The blocked particles were then centrifuged at 10,000 rpm for 15 minutes; one reaction volume of 7.5% sucrose in 0.05 m MOPS pH 7.2/0.5% kathon was added to each tube to overcoat the particles. Sonication was carried out to resuspend the particle pellets. A 1:200 dilution of each coated prep was prepared and mixed. The spectrophotometric absorbance at 650 nm was measured for each coated preparation. The percentage of solids was calculated according to the formula which utilized the absorbance at 650 nm multiplied by the factor determined from the extinction coefficient in accordance with methods known in the art for 0.8 μm particles. Thereafter, one reaction volume of 7.5% sucrose in 0.05 MOPS pH 7.2/0.5% kathon (overcoat) was added to each prep to obtain a 2% particle solids final concentration. Three μl of 10 mg/ml FD&C blue dye stock per ml of final volume for each coated particle preparation was thoroughly mixed. The preparation was stored at -20°C.

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Preparation of Anti-Canine IgE Conjugated with a Signaling Agent

For the purpose of this invention, specific binding proteins having specificity for canine IgE are used to advantage. As defined herein, a specific binding protein is a protein that specifically binds to a binding substrate; specific binding indicates a greater than 90% probability that the substances bound are members of a specific binding pair. For example, a specific binding pair can comprise an antibody and the ligand to which it has affinity. In accordance with the invention specific binding proteins can comprise a uniform composition of monoclonal antibodies, a mixture of different monoclonal antibodies, a polyclonal mixture of antibodies, and proteinaceous material which specifically binds such as a recombinant high affinity Fc epsilon receptor protein. Preferred embodiments comprised use of, the monoclonal antibody D9 (Douglas DeBoer, University of Wisconsin, Madison) or a monoclonal antibody 14K2, 1B1, or 11B11 (Idexx, Westbrook, Maine).

For convenience the D-9 will be discussed in greater detail, with all steps analogous or identical for other specific binding proteins with preparatory and assay variations as within the knowledge of those of ordinary skill in the relevant art.

The D9 antibody was derived from hybridoma clone D9 and was purified from ascitic fluid in accordance with standard methodologies. D9 or 14K2 antibody was isolated and purified as follows: Sephadex G-25 medium (Pharmacia, Uppsala) was swelled, where 50-100 grams of the medium were added to deionized water, for 3 hours at room temperature or 1 hour in a boiling water bath. The gel was then washed 3 or 4 times

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by swirling and allowing the gel to settle to the bottom, decanting the supernatant and repeating the wash until the final wash was clear. Ascitic fluid containing monoclonal antibody D9 or 14K2 can be frozen. If frozen ascitic fluid was used, the fluid was thawed and any large fibrin clots were removed. While cold, the fluid was passed by gravity through a Whatman #4 filter paper (available from Fisher Scientific, Fairlawn, NJ) and the fluid collected. To precipitate the antibodies, saturated ammonium sulfate (45%) at a volume corresponding to 81.8% of the volume of ascitic fluid was used. The saturated ammonium sulfate (SAS) and ascitic fluid were mixed at room temperature for 30 minutes, placed on ice for 1 hour, then centrifuged for 10 minutes at 10,000 rpm at 4°C. Saturated ammonium sulfate pellets may then be saved at -20°C until needed.

The SAS (45%):ascitic pellet was cut with Pierce Protein G binding buffer to 20 ml/pellet; the fluid was centrifuged at 10,000 rpm for 10 minutes at 4°C. The recovered material was filtered with a Millex HV 0.45 μ m filter (Millipore, Bedford) prior to loading antibody onto a protein G column. A large protein G chromatography column was used with an FPLC system or an appropriate peristaltic pump with in line UV monitor. The protein G column was equilibrated with Pierce protein G binding buffer. The column was pre-eluted with 0.1 m acetic acid/0.15 NaCl buffer. The protein A column was re-equilibrated with Pierce Protein G binding buffer. The filtered antibody was then loaded onto a protein G column at 1-2 ml/minute. After the antibody had been loaded onto the protein G column, the column continued to be washed with binding buffer until the UV monitor indicated a stable baseline. The antibody was eluted at 4 ml/minute with Pierce

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Protein G elution buffer. The antibody was collected with use of the UV monitor with collection starting when a 10% deflection above baseline was indicated and stopped when the indicator returned to 20% above baseline. The antibody was 5 neutralized with 1N NaOH or dibasic sodium phosphate. The purified antibody was then dialyzed by Spectropor #1 (Spectrum, Houston) (6,000-8,000 MWCO) or Spectropor #2 (Spectrum, Houston) (10,000-24,000 MWCO) dialysis tubing against 4 liters of 100 mM carbonate buffer pH 9.5. The 10 buffer was typically changed at least once. The dialysis occurred for at least 4 hours but up to overnight. The antibody was concentrated to greater than 6 mg/ml in accordance with standard methodologies. Antibody was not stored for more than 24 hours before beginning the 15 conjugation step.

Conjugating a signaling reagent to monoclonal antibody

Conjugation of a signaling reagent to a specific binding protein such as an antibody took place in accordance with 20 methodologies known in the art. Various signaling reagents could be used, such as radio-labeling or conjugating to a enzyme which will have an effect on an enzyme substrate. Enzymes used have comprised beta-galactosidase and HRPO, each 25 method for attaching a means for signaling to a specific binding protein occurring in accordance with methods known in the art. In one embodiment, monoclonal antibody D9 or monoclonal antibody 14K2 was conjugated to horseradish peroxidase. Accordingly, a Sephadex G-25 column (Pharmacia, Uppsala) was prepared with a 2.5 cm x 20 cm chromatography 30 column with flow adapters. Approximately 10 ml of gel bed was needed for each 1 ml of horseradish peroxidase (HRPO).

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applied to the column. The column was equilibrated with 5 mM acetate buffer at pH 4.5. The effluent of the G-25 column was first checked; it should match the incoming buffer pH \pm 0.1 unit.

5 HRPO was activated as follows: Approximately the same amount of HRPO was used as the amount of antibody, the HRPO amount was rounded to the nearest 20 mg. 100 mg HRPO and 100 mg sodium metaperiodate were added to separate clean tubes. The HRPO was dissolved in 5.0 ml of 5 mM acetate buffer by careful addition and was let stand 5-10 minutes without swirling; after 5-10 minutes the fluid was gently swirled to dissolve the HRPO. 100 mg sodium metaperiodate was dissolved in acetate buffer by swirling to obtain a 10.7 mg/ml stock solution; 2.5 ml of the periodate solution was added to the HRPO and mixed by swirling. The HRPO-periodate mixture was incubated for 30 minutes at room temperature in the dark. The HRPO mixture was loaded onto a G-25 column and allowed to flow into the gel bed.

20 Thereafter, 5 mM acetate buffer was carefully added to the column. The brown HRPO band was collected as it eluted from the column. A 1:10 dilution of the HRPO from the peak was made. A A280 measurement using 5 mM acetate buffer as blank was performed, and the protein concentration and yield calculated in accordance with formulae known in the art.

25 Conjugation was performed with reagent requirements as follows: The mg of HRPO required was equal to 54.6% of the mgs of antibody; the final reaction mixture contained 4 mg/ml antibody; the reactants volume equaled the mls antibody and mls HRPO; the volume of 100 mM carbonate added equaled the final volume less the reactants volume. The calculated carbonate was added to the antibody solution as was the

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calculated HRPO, and were swirled to mix. The pH should remain at 9.5 ± 0.1 . The vessel was covered and incubated at 2 hours at room temperature with no stirring. A 2 mg/ml solution of sodium borohydride was added in an amount equal 5 to the antibody content in mg $\times 0.0125$ ml/mg, where the borohydride was dissolved in deionized water, swirled and then quickly added. The mixture was incubated 15 minutes at room temperature.

10 The conjugate was then diluted to 0.5 mg/ml final concentration with conjugate diluent. The conjugate diluent was added in an amount equal to the final volume less conjugate volume. This amount was added to the antibody mixture and was swirled to mix. The pH of the conjugate concentrate was adjusted to be 7.60 ± 0.10 .

15 Various diluents were prepared for addition to the antibody-signal conjugate in order to achieve the final antibody:signal conjugate solution that was applied to a flow matrix (Tables 1 and 2). Table 1 provides the components of two diluent base formulations; Table 2 provides information 20 concerning a diluent base, the components added to a base, and the time it took for a sample to flow from a defined sample application region of a preferred embodiment of the invention to a location distal to the allergen containing region of the solid phase.

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TABLE 1

CONJUGATE DILUENT BASE

5 6615 HTWM conjugate diluent

Gentamicin	16	mg/L	16 μ g/ml
Tris	6.05	g/L	0.05 M
Tween 20	0.5	ml/L	0.05%
HI FCS	500	ml/L	50%
Blue dye	30	mg/L	30 μ g/ml
Zwittergent	0.5	g/L	0.05%
Kathon	4.5	ml/L	0.45%
HI mouse serum	100	ml/L	10%

15

Table 1 (cont.)

6680 Monoclonal conjugate diluent

Gentamicin	16	mg/L	16 μ g/ml
Tris	6.05	g/L	0.05 M
Tween 20	0.5	ml/L	0.05%
HI FCS	500	ml/L	50%
Blue dye	30	mg/L	30 μ g/ml
Kathon	30	ml/L	3%
HI mouse serum	100	ml/L	10%

20

25

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TABLE 2

	CONJUGATE DILUENT BASE	ADDITIONAL COMPONENTS	TIME TO REACH ACTIVATE WINDOW (SECONDS)
5	6680	NONE	22
	6615	PEG 1500 MW (5%) DEXTRAN SULFATE 500,000 MW (3%)	113
	6615	PEG 1500 MW (5%)	22
	6680	PEG 6000-8000 MW (5%)	28
10	6615	PEG 6000-8000 MW (5%)	29
	6680	PEG 6000-8000 MW (2.5%)	29
	6615	PEG 6000-8000 MW (2.5%)	30
	6615	DEXTRAN SULFATE 5000 MW (20%)	26
	6615	DEXTRAN SULFATE 500,000 MW (3%)	70
15	6615	DEXTRAN SULFATE 500,000 MW (3%) FICOLL 400,000 MW (5%)	90
	6615	DEXTRAN SULFATE 500,000 MW (3%) POLYVINYL PYRROLIDONE 40,000 MW (5%)	103
	PEG IS POLYETHYLENE GLYCOL		
	FICOLL IS A REGISTERED TRADEMARK OF PHARMACIA INC.		

20 A diluent for an embodiment of the method which did not perform at the limit of rapidity but had somewhat higher sensitivity was as follows: TRIS base 6.05 g/L \pm 0.3 g; kathon 4.5 ml/L \pm 0.25 ml; gentamicin sulfate 16 mg/L \pm 0.8 mg; Tween 20 (Fisher Scientific, Fairlawn) 0.5 ml/L \pm 0.025 ml; Zwittergent (Behring Diagnostics, La Jolla) 3-14 0.5 g/L \pm 0.025 g; dextran sulfate 30 g/L \pm 1.5 g; fetal bovine serum 500 ml/L \pm 25 ml; mouse serum 100 ml/L \pm 5 ml; blue dye FD&C #1 30 mg/L \pm 1.5 mg; and, sterile DI water 350 ml/L \pm 22 ml: this produced a volume of 1 L. The ingredients were combined

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and the pH adjusted to 7.6 ± 0.1 . The conjugate diluent was filtered through a $0.20 \mu\text{m}$ filter, and stored at $2-7^\circ\text{C}$.

An antibody-HRPO conjugate prepared as defined herein was further diluted in accordance with the following methodology: to determine the volume of antibody-signal conjugate to use, the volume in ml of diluted bulk conjugate desired was multiplied by the concentration in $\mu\text{g}/\text{ml}$ of the antibody-HRPO conjugate, where the concentration of conjugate was divided by $500 \mu\text{g}/\text{ml}$. The volume of antibody conjugate diluent needed was equal to the volume of the bulk conjugate in (ml) less the volume of the conjugate concentrate to use as defined in this paragraph. Accordingly, the volume of the antibody conjugate plus the volume of the antibody conjugate diluent needed was equal to the total volume. The antibody conjugate and the diluent were mixed for a minimum of 30 minutes, but less than 8 hours, at room temperature. The pH was measured at room temperature and adjusted to 7.4 ± 0.05 . The product was filtered immediately prior to placing it into containers.

20

Preparation of Wash Solutions

As a first step in selecting a preferred wash solution, it was generally attempted to chose a wash solution that had hydrodynamic properties analogous to the properties of the conjugate-sample mixture.

A wash solution for use with a slower, sensitivity-stabilized invention was composed of the following ingredients: citric acid, $0.875 \text{ g} \pm 0.05 \text{ g}$; sodium citrate $6.1 \text{ g} \pm 0.1 \text{ g}$; potassium chloride, $37.3 \text{ g} \pm 0.1 \text{ g}$; kathon, 5 ml; Triton X-100 (Sanger, St. Louis), 1.5 ml; Tween-80 (Fisher Scientific, Fairlawn), 1.5 ml; dry milk, $12.5 \text{ g} \pm 0.5$

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g; BSA (liquid-30%), 83.3 ml. To prepare the wash solution, deionized water corresponding to 70% of the desired batch size was added to a container; with constant mechanical stirring the citric acid, sodium citrate, potassium chloride, 5 kathon, Tween-80 and Triton X-100 were added and stirred until dissolved. The resulting pH should be between 4.8 and 6.8, and was not adjusted. Thereafter, with constant mechanical stirring, the dry milk and BSA were added with stirring continued, and foam formation avoided, until all 10 components were in solution. The pH was adjusted to be 6.8 ± 0.05. A sufficient quantity of deionized water was added to achieve the final batch size. The solution was stirred until dissolved and allowed to sit at room temperature at least one but less than 72 hours. The solution was then prefiltered 15 with diatomaceous earth in accordance with standard methodologies. Prefiltration, a first fine (0.2 m) filtration with final filtration were done with the solution equilibrated to room temperature. The solution was not refrigerated until after the filtration steps and vialing. 20 The prefilter could be obtained from Seitz (Seitz Filter Werke, Bad Kreuznach, Germany) (DE coating SOP MFG-16) and an intermediate filtration with a 0.65 micron filter (Cuno, Meriden, CT, part 300 PEK152165P) and a 0.2 micron final filtration. (Cuno, code 7000 3-01A-0205T). The volume of the 25 filtered wash solution was measured, and a volume of potassium chloride to be added calculated as: Number of liters of filtered wash multiplied times 37.275 g. With constant mechanical stirring the potassium chloride was added to the filtered wash, and stirred until dissolved. The pH was measured and adjusted to 6.8 if necessary. The solution 30

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was allowed to sit at room temperature at least 12 hours and up to 72 hours and was final 0.2 μm filtered before filling.

Preparation of Positive and Negative Control Particles

Positive and negative controls were prepared in accordance with the following conceptual framework: a positive control was selected that would act as a substrate for the signal substance that was conjugated to the specific binding protein; a negative control substance was chosen which was not likely to be bound by either the sample (serum or plasma) or by the specific binding protein.

To prepare positive and negative controls, various buffer solutions were prepared.

For example, a 0.01 M potassium phosphate buffer was prepared by adding potassium-phosphate monobasic 0.381 g; potassium-phosphate dibasic 1.643 g; and, 1 L of deionized water. The water was added to a container and then the other ingredients added with constant mechanical stirring; the pH was then adjusted to 7.2 ± 0.1 . Sufficient deionized water was added to achieve the desired batch size. The solution was filtered with a 0.2 μm filter.

A solution of 1% BSA in 0.01 M potassium phosphate buffer was prepared by adding 10 g of BSA with 1 L of 0.01 M potassium phosphate buffer prepared as described herein. Accordingly, the BSA was added to the potassium phosphate buffer with constant mechanical stirring until dissolved. The pH was measured and adjusted to 7.2 ± 0.1 . A sufficient quantity of deionized water added to achieve the final batch volume; the solution was then filtered through a 0.20 micron filter.

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A solution of 0.05 M MOPS buffer was prepared by adding MOPS 20.9 g \pm 0.10 g and 2 liters of deionized water. Accordingly, deionized water in a volume of approximately 70% of the batch size was added to a container, and the MOPS added with constant mechanical stirring until dissolved. The pH was adjusted to 7.2 \pm 0.05, and sufficient deionized water added to achieve the final volume. The solution was then filtered through a 0.22 micron filter.

A particle overcoating solution containing 7.5% sucrose was prepared by adding 75 g of sucrose to 1 L of 0.05 M MOPS pH 7.2 and 5 mls of kathon. Accordingly, 0.05 M MOPS in a volume of 90% of the batch size was added to a container, then the sucrose and kathon were added with constant mechanical stirring until dissolved. The solution was adjusted to 7.2 \pm 0.1, and sufficient deionized water added to achieve the batch volume. The solution was filtered through a 0.20 μm filter.

To prepare positive control particles, the following steps were undertaken. The, 0.8 μm particles were washed by adding 15 ml of 0.01 M potassium-phosphate buffer with 0.1% sodium azide at pH 7.2 to each 5 ml of 0.8 μm particles. Each tube was centrifuged then decanted and the pellet resuspended to 5 ml in the potassium-phosphate/azide buffer. A sonic probe was used to resuspend the particles. For the positive particles, the volume of stock antibody per tube (ml) was equal to (the coating concentration (mg/ml) multiplied times the coating volume per tube (ml), all divided by the concentration of stock antibody (mg/ml)). The amount of PBS to be added to each tube was calculated as equal to the coating volume less particle volume less volume of anti-HRPO antibody (which functions as the positive

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control). Accordingly, the required amount of 1X PBS was added to each tube of the particle suspension; the required amount of antibody was then added dropwise to the suspension. The tube was sonicated and checked for single particle dispersion, then incubated for 12-24 hours at room temperature on a lab shaker. The particles were then transferred to centrifuge tubes using the same number of tubes originally started with. All particles were centrifuged at 15,000-17,000 g for 10 minutes; the supernatant was carefully decanted and the pellet drained. Then, 10 ml of 1% BSA solution was added to each tube, which was then sonicated to resuspend the particles. Resuspension was verified under a microscope to identify uniform particle dispersion. The solution was then incubated on an orbital shaker for 1 hour at room temperature.

To overcoat the positive particles, the particles were centrifuged for 10 minutes, the supernatant decanted and the pellets drained; 20 ml of the 0.01 M potassium phosphate solution was added to each tube and the pellets resuspended by sonication. The particles were centrifuged at 15,000-17,000 g for 10 minutes, the supernatant decanted and the pellets drained. Thereafter, 20 ml of the 7.5% sucrose overcoating solution was added to each tube and the particles resuspended by sonication. The particles were then pooled in appropriate bottles and particle dispersion checked microscopically. The percentage of solids was adjusted as described below.

To prepare negative particles, the volume of 0.8 micron BSA coated polystyrene particles needed was equal to twice the batch size. The BSA coated particles were dispensed into 500 ml Nalgene (Nalge Co., Rochester), bottles and

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centrifuged at 15,000-17,000 g for 10 minutes, the supernatant then carefully decanted and the pellet drained. The amount of 7.5% sucrose for resuspension of particles to achieve approximately 3% solids was calculated as volume of the BSA coated 0.8 micron particles dispensed divided by three. The calculated amount of 7.5% sucrose was added, and the particles resuspended by sonication, then checked microscopically for uniform particle dispersion. The particles were pooled.

The positive and negative control particles were adjusted as to percentage solids. The positive particles were adjusted to 1% \pm 0.05% solids and the negative particles were adjusted to 2% \pm 0.05% solids by use of the 7.5% sucrose solution. The optical density of 1:200 dilutions of positive and negative particles were obtained at 650 nm. The particles were then diluted with sucrose overcoat buffer according to the following formula: $B \div (C \times A) = D$; $D - A = E$ where A = original volume; B = original % solids; C = optimal % solids; D = final volume; and, E = volume of sucrose; where the % optimal solids = 1 for positive particles and = 2 for negative particles. The percentage of solids were determined in accordance with standard methodologies.

The positive and negative particles were stored at 2-7°C if stored less than 14 days, and at -20°C in 10 ml aliquots if stored for longer than 14 days.

Spotting of Allergen-coated 0.8 μ m Particles and Control Substances onto a Flow Matrix

The placing of an aliquot of allergen-coated particles or control particles on a matrix is referred to as spotting.

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To achieve spotting onto a flow matrix an apparatus such as a pipette was used. Before spotting, an apparatus which was to be used for dispensing the particles was first primed with 0.1% Tween-water solution for 15 minutes, primed with sterile deionized water for 15 minutes, then primed with overcoating solution for 10 minutes. Prior to spotting, particles were sonicated to eliminate aggregation. The particles were checked periodically at the dispensing tip of the apparatus and were only sonicated if aggregation was present; the particle suspension should be maintained throughout the spotting process, such as by application of shaking forces. Typically, spots were made on the matrix by use of 1.2 μ l of a particle-containing fluid or a control fluid. The tip of a dispensing apparatus did not contact the flow matrix when the particles were being spotted. Following spotting, the matrix is thoroughly dried. Thereafter, the matrix was packaged in an airtight container that preferably contained a desiccant.

The polystyrene 0.8 micron particles were originally in a sucrose solution; following spotting onto the flow matrix the particle-sucrose solution was allowed to dry. Thereafter, even though the spot can be saturated with fluids, such as the reagents, the 0.8 micron particles remain attached to the flow matrix and were not dispersed. It is believed that the factors accounting for this may include a tendency for particle aggregation, possibly due to interparticle charge factors. The 0.8 micron particles were commercially obtained (Seradyn).

In order to enhance the visibility of the assay materials during the performance of a test, FD&C blue dye at a concentration of 6.0 mg/ml was prepared in accordance with standard methodologies by dissolving sufficient blue dye

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5 powder in DIH₂O. To produce a reference dye, 10 ml of the 6.0 mg/ml concentrated blue dye was diluted with 90 ml deionized water and mixed well. In one embodiment, this dye can be placed at a location distal to the region of the matrix where the allergen-bound particles are spotted. The appearance of the blue dye at the surface of a flow matrix when the sample liquid arrives at this distal location indicates that the sample fluid has moved past the region containing the allergens. Thus, the use of blue dye helps an operator 10 identify fluid permeation.

System Operation

15 In one embodiment of a system in accordance with the invention a flow matrix was used. As used herein a flow matrix is preferably one that is that is hydrophilic, and permeable fluids. Examples of effective flow matrices include but are not limited to membranes, cellulose acetate, glass fiber membranes, cellulose matrices such as paper or sponge. A preferred flow matrix was a permeable flow matrix 20 comprising scintered polyethylene particles (Chromex, Interflow, Brooklyn) was used. A monoclonal antibody - HRPO conjugate was prepared as described herein. A standard precipitating substrate for the HRPO enzyme was commercially obtained (MOSS, Inc., Pasadena MD).

25 In accordance with a preferred embodiment, canine serum or plasma, either fresh, previously frozen, or stored at 2-7°C was used. Serum or plasma was stored up to 7 days at 2-7°C. Previously frozen or refrigerated samples were allowed to come to room temperature before use. It was found that 30 EDTA or heparin in plasma did not affect the results; and

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hemolyzed samples did not affect the results. Previously frozen or older samples were centrifuged before use.

Serum or plasma (200 μ l) was mixed with a corresponding volume of the final solution of specific binding protein conjugated to a signal. The combination of serum or plasma with monoclonal antibody conjugate was then placed onto a flow matrix to which had been previously spotted 0.8 μ polystyrene particles to which were attached a canine allergen and/or control particles. The fluid containing serum or plasma combined with conjugate was allowed to flow past the spots. Thereafter, the wash solution was added to diminish the presence of serum or plasma and free conjugate in the area of the matrix to which the allergen or control was spotted. The substrate for the signaling agent was then added to the matrix and allowed to flow to the region of the matrix on which the allergen coated polystyrene particles had been spotted. (If an embodiment is used where the specific binding proteins are conjugated to a radiolabel, this step is not necessary.) The substrate came into contact with the allergen-spotted region and produced a sensible signal within 7 minutes.

Preferably, the wash solution and the substrate solution were placed into contact with a flow matrix simultaneously, with a physical orientation of contact of these solutions to the flow matrix such that the wash solution was placed at a location proximal to the region having the spotted allergen and the substrate solution was relatively distal to the region having the spotted allergen.

In a not optimally rapid embodiment, the time course for a reaction occurring on the flow matrix was as follows: 1-2 minutes flow time, the time from placing the combination of

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sample (serum or plasma) with conjugate on the flow matrix until the combination had flowed beyond the region to which the allergen had been spotted; and a signal development period of approximately 6-7 minutes, the time from when HRPO substrate was placed on the matrix until a sensible signal was produced; thus the total time for an assay was frequently from 7-8 minutes.

5 The present invention was also used where the allergen was in a liquid phase, or where the allergen was in contact
10 with a nonpermeable solid phase surface.

Allergy Test Kits

15 The present invention can be packaged in kit form, to be conveniently used by a veterinary care professional. For example, a kit can comprise a spotted matrix which comprises a spot of allergen-coated particles and/or a spot of control particles.; and specific binding protein-signal agent conjugate. The conjugate can be in a form ready for use in the assay or can be concentrated requiring dilution prior to
20 use. In a preferred embodiment, a kit is available from IDEXX Laboratories as "CANINE ALLERGEN SPECIFIC IgE TEST KIT"; this kit comprises a SNAP™ produced in accordance with U.S. patent application Serial No. 07/738,321, filed 31 July 1991, and PCT application PCT/US92/06384 filed 31 July 1992,
25 each of which are incorporated by reference herein.

Comparison of Monoclonal Antibody Specific to Canine IgE and Polyclonal Serum Understood to be Directed to Canine IgE

30 Protein G is known to bind with several species of IgG antibody, but it does not bind IgE. To evaluate the

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sensitivity of the polyclonal serum standard in the art as being directed to Canine IgE, protein G was used.

5

TABLE 3

PATIENT 1			
Allergen	Neat	PrG absorbed	PrG eluate
Bermuda	40	1	42
Johnson	58	2	61
Kentucky Blue	43	1	49
Perennial Rye	48	1	57
Quack/Couch	37	1	43
Ragweed	40	1	53
Dandelion	59	2	76
Goldenrod	41	1	51
Hickory/Pecan	38	2	43
Red Cedar	44	1	52
Oak	38	0	46
Maple/Box Elder	48	0	44
Elm	40	2	49
E. Cottonwood	56	1	62
Ash	46	2	48
Sage	32	2	38
Mulberry	57	3	58
E. Sycamore	40	0	54
Walnut	28	2	39
Pine	76	4	83
Dust Mite	93	14	82
House dust	74	2	74
Lambs Quarter	47	9	58
Pigweed	53	1	61
Cocklebur	38	5	46
Dock/Sorrel	48	1	43
English Plantain	53	1	60
Meadow Fescue	38	8	50
Alternaria	67	0	68
Aspergillus	84	1	84
Penicillium	120	1	120

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PATIENT 1			
Allergen	Neat	PrG absorbed	PrG eluate
Cladosporium	65	0	77
Pyrethrum	58	4	81
Flea	112	3	111

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TABLE 3, (CONT.)

PATIENT 2			
Allergen	Neat	PrG absorbed	PrG eluate
Bermuda	88	4	81
Johnson	119	8	122
Kentucky Blue	109	5	107
Perennial Rye	114	6	120
Quack/Couch	106	5	111
Ragweed	106	3	95
Dandelion	133	2	89
Goldenrod	108	2	100
Hickory/Pecan	116	2	64
Red Cedar	103	0	79
Oak	82	1	76
Maple/Box Elder	105	2	106
Elm	100	2	58
E. Cottonwood	96	3	75
Ash	91	2	72
Sage	88	3	92
Mulberry	3	2	66
E. Sycamore	106	0	84
Walnut	81	1	57
Pine	118	4	84
Dust Mite	90	20	77
House dust	78	7	73
Lambs Quarter	94	3	105
Pigweed	114	3	93
Cocklebur	106	4	106
Dock/Sorrel	119	2	103
English Plantain	121	5	96
Meadow Fescue	85	5	84
Alternaria	29	0	54
Aspergillus	52	0	36

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PATIENT 2				
	Allergen	Neat	PrG absorbed	PrG eluate
10	Penicillium	61	1	49
	Cladosporium	81	2	101
	Pyrethrum	101	2	74
5	Flea	34	1	35

TABLE 3 (CONT.)

PATIENT 3				
	Allergen	Neat	PrG absorbed	PrG eluate
10	Bermuda	103	4	104
	Johnson	115	10	119
15	Kentucky Blue	117	8	119
	Perennial Rye	115	6	119
	Quack/Couch	119	4	114
	Ragweed	118	1	123
20	Dandelion	107	1	113
	Goldenrod	112	1	131
	Hickory/Pecan	88	0	99
	Red Cedar	147	3	143
	Oak	103	12	107
25	Maple/Box Elder	115	2	121
	Elm	64	1	75
	E. Cottonwood	99	1	111
	Ash	89	1	101
	Sage	93	1	104
30	Mulberry	80	2	91
	E. Sycamore	108	0	100
	Walnut	65	1	68
	Pine	115	21	131
	Dust Mite	101	53	134
	House dust	92	23	78
35	Lambs Quarter	119	1	131
	Pigweed	112	0	131
	Cocklebur	129	1	134
	Dock/Sorrel	149	8	147
	English Plantain	111	1	111
40	Meadow Fescue	104	8	105
	Alternaria	53	1	59

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PATIENT 3			
	Neat	PrG absorbed	PrG eluate
Allergen			
Aspergillus	52	0	55
Penicillium	52	1	37
Cladosporium	30	0	41
Pyrethrum	81	1	105
Flea	36	0	45

Table 3 presents data for use of an ELISA assay commercially performed by a leading, perhaps the current market leader, in the field of commercial in vitro testing for canine allergy. In Table 3, the column labeled "Neat" relates to results of the commercial ELISA where the numerical values correspond to a percentage of reactivity relative to the samples of a pooled positive control (i.e., allergic) population. In the ELISA the results were obtained by use of the polyclonal serum understood to be reactive to canine IgE. The column labeled "PrG Absorbed" indicates the value from the ELISA following absorption of the serum from each of patients 1, 2 and 3 prior to performing the ELISA. The results for each of patients 1, 2 and 3 indicate substantial decreases in results from the ELISA. The basis for this finding was as follows: Protein G binds and removed IgG, the substantial decrease in assay results showed that the polyclonal serum which had been understood to be reacting to IgE had actually been reacting with IgG present in canine serum. This result was verified by eluting the material bound to the Protein G column, then reacting the eluate with the commercial polyclonal serum, whereupon it was found that there was much reactivity. When an analogous method of contacting with Protein G was performed with the specific binding proteins in accordance with the present invention, the results established that canine IgE were being bound by

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the specific binding proteins of the invention (Figure 1); Fig. 1 exemplifies the results of a subject assay on the serum of patient 1, where the serum was put into contact with American Elm allergen, in accordance with the invention.

5 Contrary to the results with the polyclonal serum it was found that contact with protein G increased the reactivity of the present assay with either D9 or 14K2 antibodies; this result is believed to be due to removal of contaminants in the serum.

10 All publications mentioned herein are incorporated herein by reference to describe and disclose specific information for which the reference was thus discussed. It is to be noted that as used herein and in the appended claims, the singular forms "a" and "the" include plural referents unless the context clearly indicates otherwise.

15 Thus, for example, reference to "a formulation" includes mixtures of different formulations and reference to "the method of treatment" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

20 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skilled in the art. Although methods and materials or equivalent to those described herein can be used in the practice for testing of the invention, the preferred methods and materials are described herein. It is understood 25 that the invention is limited solely by the appended claims.

WHAT IS CLAIMED IS:

1. A specific binding protein that specifically and/or sensitively binds to canine IgE.
2. The specific binding protein of claim 1 which comprises a monoclonal antibody D9, 14K2, 1B1, or 11B11.
3. The specific binding protein of claim 1 which further comprises a means for producing a signal.
4. A plurality of specific binding proteins in accordance with claim 1.
5. The plurality of specific binding proteins of claim 4 which comprises a polyclonal serum reactive to canine IgE.
6. Use of specific binding proteins of claim 1 to assay for the presence of allergen-specific canine IgE, said method comprising:
 - obtaining serum or plasma from a dog;
 - contacting said serum or plasma with a canine allergen so that allergen-specific canine IgE become bound to the allergen;
 - then introducing to the allergen, specific binding proteins that sensitively and specifically bind to canine IgE, said specific binding proteins comprising a means for producing a signal; and,
 - identifying the presence of the signal, whereby the presence of the signal indicates the presence of canine IgE bound to the allergen.

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7. The method of claim 6 wherein the step of introducing to the allergen comprises introducing a means for producing a signal that produces a signal following activation or following action on a substrate; and, further comprising a step of producing a signal with the means for producing a signal.

8. The method of claim 6 wherein the step of introducing to the allergen comprises introducing a specific binding protein that comprises a monoclonal antibody D9, 14K2, 1B1, or 11B11.

9. The method of claim 8 wherein the step of introducing to the allergen comprises introducing a specific binding protein that is monoclonal antibody D9, 14K2, 1B1, or 11B11.

10. The method of claim 6 wherein the contacting step comprises contacting with a canine allergen that is itself in contact with a flow matrix.

11. The method of claim 10 wherein the allergen is bound to the solid phase by covalent binding.

12. The method of claim 6 further comprising a step of washing nonbound material from the allergen following the contacting step.

13. The method of claim 7 wherein the step of identifying the presence of a signal comprises identifying within 300 minutes of the step of producing a signal.

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14. The method of claim 13 wherein the step of identifying the presence of a signal comprises identifying within 10 minutes of the step of producing a signal.

15. The method of claim 6 further comprising a step of conducting an assay for the presence of allergen-specific IgG by use of a polyclonal serum reactive to IgG.

16. Use of the specific binding protein of claim 1 to assay for the presence of allergen-specific canine IgE, said method comprising:

obtaining serum or plasma from a dog;

contacting said serum or plasma with a canine allergen immobilized on a solid phase whereby an IgE in the serum will bind to the allergen;

washing nonbound material from the allergen;

contacting the allergen with a specific binding protein that comprises a means for signaling, wherein the specific binding protein specifically binds canine IgE; and,

evaluating for the presence or absence of the means of signaling of the specific binding protein bound to the canine IgE bound to the allergen, so as to identify the presence of allergen-specific canine IgE.

17. The method of claim 16 wherein the step of contacting the allergen comprises contacting with a specific binding protein that comprises a means for signaling that causes a signal following activation or following action on a substrate; and,

further comprising a step of producing a signal with the means for producing a signal.

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18. The method of claim 16 wherein the step of contacting the serum or plasma comprises contacting with a canine allergen immobilized on a flow matrix.

19. Use of the specific binding protein of claim 1 to assay for the presence of allergen-specific canine IgE, said method comprising:

obtaining serum or plasma from a dog;

providing a solid phase flow matrix capable of driving fluid movement, said matrix comprising a region which comprises immobilized canine allergen;

providing a mobile disclosure reagent which can move through the matrix, wherein the mobile disclosure reagent comprises specific binding proteins that specifically bind canine IgE;

providing a washing liquid which can move through the allergen immobilized on the solid phase matrix;

contacting the allergen with the mobile disclosure reagent, wherein said disclosure reagent is detectible;

removing material nonbound to the allergen from the region comprising immobilized allergen by contacting the allergen with the washing liquid; and,

detecting the presence of the disclosure reagent attached to the canine IgE which is attached to allergen, thereby identifying allergen specific canine IgE.

20. The method of claim 19 wherein the step of providing a matrix provides a solid phase flow matrix comprising two matrix regions:

(i) a region comprising immobilized canine allergen; and,

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(ii) a receipt region, said receipt region comprising a region for receipt of the serum or plasma, and a region for receipt of the mobile disclosure reagent and receipt of the washing liquid.

21. The method of claim 19 wherein the step of providing the washing liquid is the step of providing the mobile disclosure reagent.

22. A kit for use to facilitate the diagnosis of allergy in dogs, said kit comprising:

a solid phase material comprising a canine allergen in contact therewith; and,

the specific binding protein of claim 1 conjugated with a means for signaling.

23. The kit of claim 22 wherein the solid phase material is a flow matrix.

24. The kit of claim 22 wherein the solid phase further comprises a positive or negative control substance for the means for signaling.

25. The kit of claim 22 wherein the specific binding proteins conjugated with the means for signaling are in a liquid suspension or a liquid solution.

26. The kit of claim 25 wherein the specific binding proteins conjugated with the means for signaling are fully diluted for use in an assay in accordance with the kit.

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27. A method to facilitate diagnosis of allergy in a dog, said method comprising:

performing an assay on canine serum or plasma to sensitively and/or specifically identify the presence of allergen-specific canine IgE within 300 minutes of initiation of performance of the assay; and,

identifying the presence of allergen-specific canine IgE.

28. The method of claim 27 wherein the step of performing an assay comprises the binding of a monoclonal antibody sensitive and specific for canine IgE to canine IgE.

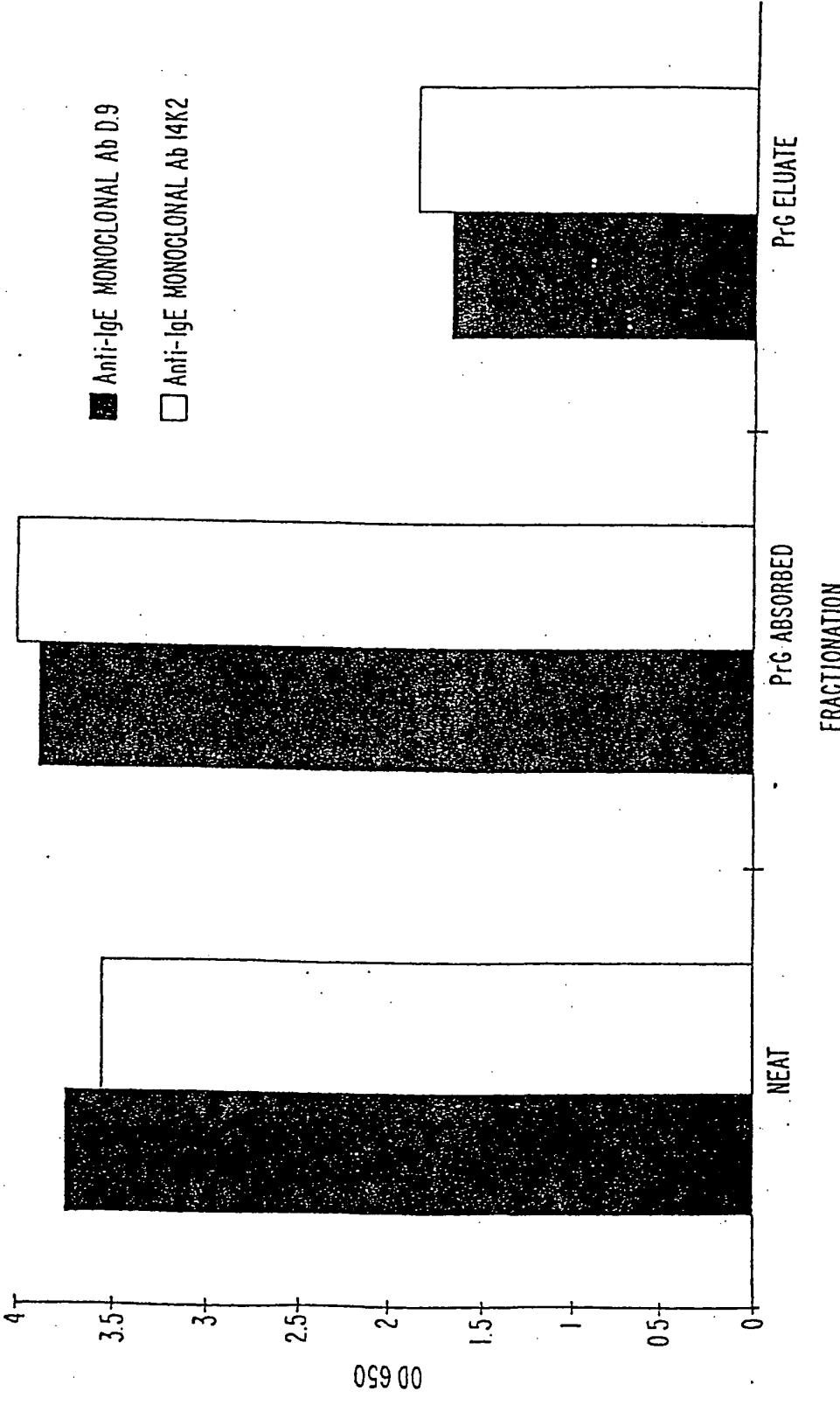
29. The method of claim 28 wherein the identifying step comprises materials in the following orientation: a solid phase, an allergen adherent to the solid phase, canine IgE specifically bound to the allergen, and, a specific binding protein comprising a means for signaling specifically bound to the canine IgE.

30. The method of claim 27 wherein the performing step comprises performing an assay on the serum or plasma to sensitively and specifically identify the presence of allergen-specific canine IgE within 10 minutes of initiation of performance of the assay.

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FIG. 1.

SIGNAL vs. FRACTIONATION - IDEXX ALLERGEN SPECIFIC IgE ASSAY
 20 μ l SAMPLE, AMERICAN ELM PLATE



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19349A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/42 G01N33/558 G01N33/577 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOLOGY, vol. 85, no. 3, July 1995, OXFORD, GB, pages 429-434, XP002029127 D. GEBHARD ET AL.: "Canine IgE monoclonal antibody specific for a filarial antigen: Production by a canine x murine heterohybridoma using B cells from a clinically affected lymph node." see page 431, left-hand column, line 8 - line 22 see figure 3	1-9, 12-17, 22,24-30
Y	---	10,11, 18-21,23
Y	WO 93 03176 A (IDEXX LABORATORIES, INC.) 18 February 1993 cited in the application see claims ---	10,11, 18-21,23
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 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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1

Date of the actual completion of the international search

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Nooij, F

INTERNATIONAL SEARCH REPORT

Intern'l Application No
PCT/US 96/19349

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AMERICAN JOURNAL OF VETERINARY SCIENCE, vol. 54, no. 2, February 1993, SCHAUMBURG, USA, pages 239-243, XP000647982 Z. PENG ET AL.: "Measurement of ragweed-specific IgE in canine serum by use of enzyme-linked immunosorbent assays, containing polyclonal and monoclonal antibodies." see the whole document ---	1,3-7, 12-17, 22,24-30
X	INTERNATIONAL ARCHIVES OF ALLERGY AND IMMUNOLOGY, vol. 102, no. 2, 1993, BASEL, SWITZERLAND, pages 176-184, XP000647986 Z. PENG ET AL.: "Purification and identification of polyclonal IgE antibodies from ragweed-sensitized dog sera." see abstract see page 178, left-hand column, line 14 - right-hand column, line 41 ---	1,3-5
X	AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 55, no. 7, July 1994, SCHAUMBURG, USA, pages 944-948, XP000647983 P. HILL ET AL.: "Quantification of serum total IgE concentration in dogs by use of an enzyme-linked immunosorbent assay containing monoclonal murine anti-canine IgE." cited in the application see abstract ---	1-3
X	VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, vol. 37, no. 3-4, August 1993, AMSTERDAM, NL, pages 183-199, XP000647974 D. DEBOER ET AL.: "Production and characterization of mouse monoclonal antibodies directed against canine IgE and IgG." cited in the application see the whole document ---	1-4
X	VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, vol. 44, no. 2, January 1995, AMSTERDAM, NL, pages 105-113, XP000647975 P. HILL ET AL.: "Concentrations of total serum IgE, IgA, and IgG in atopic and parasitized dogs." see abstract see page 107, line 29 - line 41 ---	1,3,4
1		-/-

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19349

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 550 020 A (TANOX BIOSYSTEMS, INC.) 7 July 1993 see examples see claims -----	1
P,X	KLEINTIERPRAXIS, vol. 41, no. 9, September 1996, SWITZERLAND, pages 643-650, XP000647058 B. BIGLER ET AL.: "Messung von allergenspezifischem IgE beim Hund mit einem in-vitro-Test mit monoklonalem anti-IgE-Antikörper. Vergleich mit dem Intrakutantest und erste Ergebnisse der Hyposensibilisierung." see page 644, left-hand column, line 23 line 53 -----	1-9, 12-17, 22,24-30

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/19349

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9303176 A	18-02-93	CA 2113351 A EP 0600929 A JP 6510602 T	18-02-93 15-06-94 24-11-94
EP 550020 A	07-07-93	JP 5199895 A CA 2086131 A ZA 9210006 A	10-08-93 25-06-93 29-06-93

